Design and engineering of human TRAIL variants

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Stabilization of TRAIL, an all β-sheet multimeric protein, using computational redesign.

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Abstract

Protein thermal stability is important for therapeutic proteins, both influencing the pharmacokinetic and pharmacodynamic properties and for stability during production and shelf-life of the final product. In this study we show the redesign of a therapeutically interesting trimeric all beta-sheet protein, the cytokine TRAIL, yielding variants with improved thermal stability. A combination of TNF ligand family alignment information and the computational design algorithm, PERLA, were used to propose several mutants with improved thermal stability. The design was focused on non-conserved residues only, thus reducing use of computational resources. Several of the proposed mutants showed a significant increase in thermal stability as experimentally monitored by far-UV CD thermal denaturation. Stabilization of the biologically active trimer was achieved by monomer subunit or monomer-monomer interface modifications. A double mutant showed an increase in apparent T_m of 8 ^oC in comparison to rhTRAIL WT and remained biologically active after incubation at 73 ^oC for 1h. To our knowledge, this is the first study that improves the stability of a large multimeric β-sheet protein structure by computational redesign. A similar approach can be used to alter the characteristics of other multimeric proteins, including other TNF ligand family members.

Introduction

Besides influencing the final pharmacokinetic and pharmacodynamic properties of a protein therapeutic, stability is also important throughout the production process and for the shelf-life of the final product[156]. Several strategies are used to augment the thermal stability of proteins[50,51]. Both rational[52-55] and directed evolution methods[56-58] have been successfully used to improve stability. A disadvantage of a rational approach is that one can design only a limited number of potentially improved variants. In contrast, directed evolution methods allow large numbers of variants to be generated and screened. However, suitable selection/screening procedures are required, which are often not available or are very labour intensive. More recently, computational redesign algorithms have been employed to enhance stability, amongst other properties, of proteins[59-61,157]. These methods combine computer design steps with *in silico* screening, permitting screening of a much larger sequence space than is experimentally possible with high-throughput techniques. Efficient algorithms are needed to search the vast sequence space and accurate scoring functions are required in order to rank the best designs[39,42]. Recently, computational redesign has been used to generate a hyper-thermophilic variant of streptococcal Gβ1 domain protein[59], to enhance the stability of the spectrin SH3 domain[60] and to improve the (thermal) stability of the therapeutically interesting four helix bundle cytokines, granulocyte-colony stimulation factor (G-CSF)[63] and human growth hormone (hGH)[64].

In this study, we use the automated computer algorithm PERLA[158,159] and the empirical forcefield FOLD-X[160] to improve the thermal stability of a multimeric all β-sheet protein, tumor necrosis factor-related apoptosis inducing ligand (TRAIL; TNFSF10)[161,162]. TRAIL is a member of the tumor necrosis factor ligand family. Ligands belonging to this family are involved in a wide range of biological activities, ranging from cell proliferation to apoptosis, and they share similar structural characteristics. All monomeric subunits of these ligands consist of antiparallel β-sheets, organized in a jellyroll topology, and these subunits self associate in bell-shaped homotrimers, the bioactive form of the ligand. Sequence homology is highest between the aromatic residues responsible for trimer formation. A trimer binds three subunits of a cognate receptor, each receptor subunit binding in the grooves between two adjacent monomer subunits. The ligands are type II transmembrane proteins, but the extracellular domain of some members can be proteolytically cleaved from the cell surface, yielding a bioactive soluble form of the ligand. Recent reviews of the TNF ligand-family are readily available[1,2].

TRAIL in its soluble form selectively induces apoptosis in tumor cells *in vitro* and *in vivo*, by a death receptor mediated process[19]. Unlike other apoptosis-inducing TNF family members, it appears to be inactive against normal healthy tissue, therefore attracting great interest as a potential cancer therapeutic[13]. Several crystal structures of TRAIL[17,163] and TRAIL in complex with the death receptor 5 (DR5)[12,16,18] are available. Unlike other TNF family members TRAIL has a zinc binding site in its trimeric core and the presence of the zinc ion is known to be vital for the trimeric structure and bioactivity[17,164]. Several versions of recombinant soluble TRAIL with different N-terminal fusions tags have been reported, however these versions appear to have different bioactivity profiles in comparison to the

non-tagged 'wild-type' soluble TRAIL encoding amino acids 114-281[105]. The increased *in vitro* toxicity towards certain normal healthy cells is especially noticeable in the presence of exogenous tags[14]. We therefore chose to increase the stability of TRAIL by modification of the soluble ligand version (114-281), without addition of any exogenous tags. In view of a possible use as a therapeutic protein; a close resemblance to the wild-type structure is desirable. To our knowledge, this is the first study that shows improvement of the stability of a large multimeric protein structure by computational redesign. Methods used in this study are also applicable to other TNF family ligands.

Results

Computer screening.
Novel mutants of TRAIL have been designed in order to increase the stability of the bioactive trimer. Predictions were based on the automated computer algorithm, PERLA[158,165], as described in the methods section. Briefly, the program performs strict inverse folding: a fixed backbone structure is decorated with amino acid side chains from a rotamer library. Relaxation of strain in the protein structure is achieved via the generation of subrotamers. Most terms of the scoring function are balanced with respect to a reference state, to simulate the denatured protein. The side chain conformers are all weighted using the mean-field theory and finally candidate sequences with modelled structures (PDB coordinates) are produced. Energy evaluation of the modelled structures was carried-out by a modified version of FOLD-X[150,160], available at (http://fold-x.embl-heidelberg.de). The force field module evaluates the properties of the structure, such as its atomic contact map, the accessibility of its atoms and residues, the backbone dihedral angles, in addition to the H-bond network and electrostatic network of the protein. The contribution of water molecules making two or more H-bonds with the protein is also taken into account. The algorithm then proceeds to calculate all force field components: polar and hydrophobic solvation energies, van der Waals' interactions, van der Waals clashes', H-bond energies, electrostatics, and backbone and side chain entropies.

Selection of the template sequence
The template selected was 1DU3[12]. The crystal structure at 2.2 Å resolution contains the trimeric structure of human TRAIL in complex with the ectodomain of the DR5 receptor. The TRAIL monomer lacks an external, flexible loop (130-146), not involved in receptor binding or in monomer-monomer interaction. To complete the molecule, this loop was modeled using the structure of 1D4V (2.2 Å)[18], a monomeric TRAIL in complex with DR-5 receptor, having the atomic coordinates of the loop. Finally, the TRAIL molecule was isolated by removing the receptor molecules from the PDB file.

Computational design of mutants
The visual inspection of the isolated monomers, monomer-monomer interface and central core of TRAIL showed several residues as potential candidates for mutagenesis. The highly conserved hydrophobic residues were discarded from this list. After generating the mutants we identified if there were residues involved in receptor binding. These residues in principle could not be mutated without disrupting interactions with the receptor. However, it could be that a small decrease in binding affinity could be compensated by an increase in

stability. Thus one TRAIL variant (M2), that showed a significant predicted increase in stability but also contained residues involved in receptor interaction, was retained for subsequent experimental analysis.

Table 1. Residues initially considered for design

Monomer Set	**Dimer Set**	**Trimer Set**	**Misc. Set**
E194†	H125	R227†	A123
I196†	F163	C230	A272
	Y185	Y240	S225†
	Q187		V280
	S232		F163
	D234		A123
	Y237† (D203,Q205)		V208
	L239		
	S241		
	E271†		
	F274		

† Used in subsequent rounds of design

Mutants in parenthesis were added in subsequent rounds as interaction partners

The sequence space search for every position was simplified by checking the naturally occurring amino acids in a multiple sequence alignment of proteins belonging to the TNF ligand family, thus decreasing the computing time, and subsequently focusing on non-conserved residues. The use of protein rational design and force field algorithms allowed the identification of a list of mutant sequences with potential relevance for TRAIL stability. Four sets of residues were selected for design (Figure 1b and Table 1): (1) non-conserved residues at the surface of the monomer ('monomer' set), (2) non-conserved residues near positions close to the interface between two monomers ('dimer' set), (3) non-conserved residues along the central trimeric axis ('trimer' set) and (4) a miscellaneous set ('misc. set'). The automated computer design algorithm was applied as previously described[166]. Amino acid substitutions were introduced at the non conserved residue positions in conformations (side chain rotamers) compatible with the rest of the structure. Subsequently, favorable mutations were combined and evaluated in terms of free energy (kcal mol^{-1}), and unfavorable combinations (e.g. high Van der Waal clashes) were eliminated. An output of sequences and coordinates was produced and ranked in terms of free energy and subsequently reintroduced in the design algorithm for a 2^{nd}, 3^{rd} or 4^{th} round of design, if

necessary. Table 1 summarizes the list of mutants assayed *in silico* for increased stability of TRAIL. Some of these predictions were discarded directly after theoretical energy calculations, without further experimental analysis.

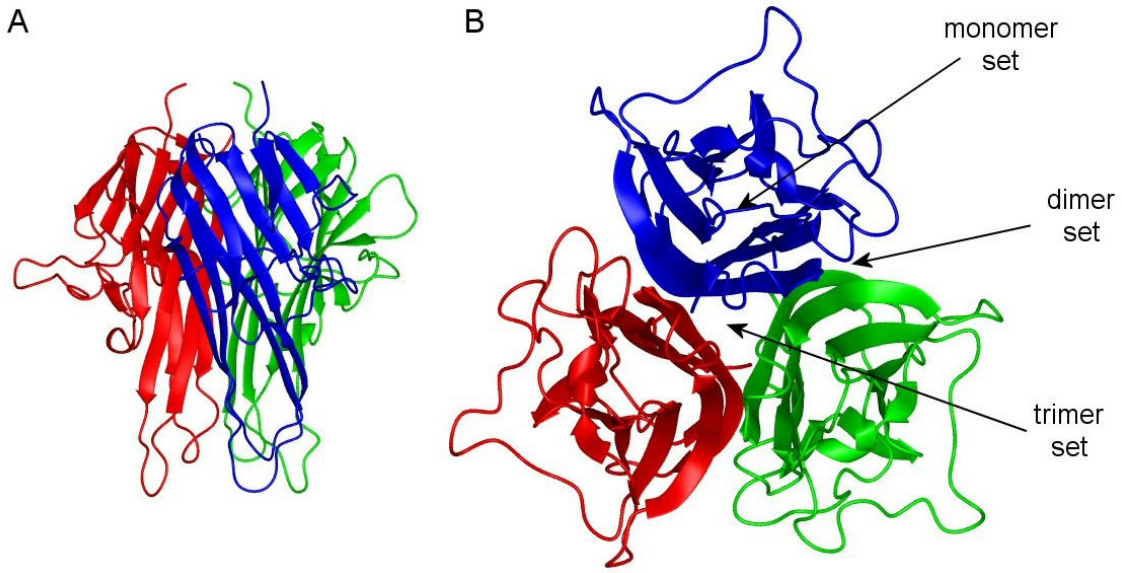

Figure 1. A) Side view of the TRAIL trimeric complex, showing the three monomers in red, blue and green. B) Top view of the same complex but viewed along the longitudinal axis, depicting the different sets used for design. Structure figures were generated using MOLMOL[167].

Description of the tested mutations

Predicted mutants were energy minimized and subsequently analyzed with FOLD-X. The energy values obtained were compared to that of the wild-type structure and used for discrimination of candidates. Mutants were selected based on an improvement in free energy relative to TRAIL WT (Table 2). In the monomeric set, M1 (E194I, I196S) was selected because of the large improvement of energy compared to TRAIL WT ($\Delta\Delta G$ = -9.7 kcal mol^{-1} $monomer^{-1}$). This low energy value is due to the fact that a trimer is being studied, in addition to the presence of significant van der Waals' clashes in the crystal structure (~5 kcal mol^{-1} $monomer^{-1}$), which are removed upon mutation. The mutations are located in the external loop connecting the C and D anti-parallel beta strands (CD loop), following the notation according to Eck[168]. The predicted increase in stability of M1 can be explained since Glu 194 is surrounded by hydrophobic groups (Trp 231, Phe 192, Ala 235) and the carboxyl group is uncompensated. The mutation Glu 194 to Ile rectifies this situation by replacing the charged residue for a medium sized hydrophobic residue. Conversely, Ile 196 is surrounded by polar residues (Asn 202, Lys 233) and is very close to the backbone, resulting in probable van der Waals clashes. Mutation to Ser avoids clashes and allows formation of a hydrogen bond to Asn 202, located in the opposite part of the CD loop (Figure 2a). Both mutations improve polar solvation energy, in addition to ameliorating side chain and backbone entropy.

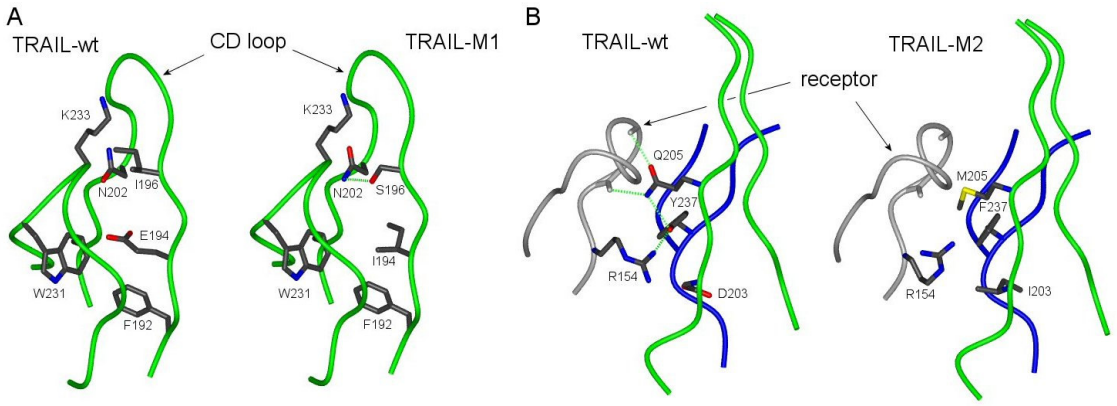

Figure 2. A) Comparison, between rhTRAIL WT and M1, of the local environment around residues 194 and 196. B) Comparison between rhTRAIL WT and M2. Backbones of the two adjacent monomers are in green and blue, respectively, and the backbone of the DR5 receptor is in grey. Hydrogen bond interactions are depicted in dashed green lines.

In the dimeric set (Table 2), the design of M2 (D203I, Q205M, Y237F) leads to the creation of a hydrophobic cluster to stabilize the interaction between residues 203 and 205 (D strand) of one monomer, and residue 237 (F strand) of the adjacent monomer. Gln 205 and Tyr 237 together form an intermolecular hydrogen bond, and Asp 203 points to a gap in the monomer-monomer interface. Mutation to Ile (203), Met (205) and Phe (237) breaks the Q205-Y237 hydrogen bond, but facilitates the tight packing of these residues, improving van der Waals interactions, hydrophobic and polar solvation energies of the entire TRAIL molecule, without a further increase of van der Waals clashes (Figure 2b). Although FOLD-X predicted that the affinity of M2 for the DR5 receptor is lower ($\Delta\Delta G_{binding}$=7.3 kcal mol^{-1} monomer^{-1}) than for TRAIL WT, this mutant was retained as a control to evaluate the accuracy of the procedure.

Residue 225 of M3 (S225A), belonging to the 'Miscellaneous set', is located in strand E and is solvent exposed in the monomeric form. However, after trimerization, this position becomes buried in a small pocket, leaving the side chain of the hydrogen bond donor Ser uncompensated. After mutation to Ala, the energy of the model is better than TRAIL WT for both polar and hydrophobic solvation energies, in addition to side chain entropy.

The Arg 227 residues of the trimeric set mutant (M4) are located in strand E, equidistantly opposed in a central position along the longitudinal axis of the TRAIL trimer. The three arginines are surrounded by hydrophobic (Ile242), polar (Ser241, Ser225) and aromatic (Tyr 240, Tyr 243) residues. These tyrosines direct the hydroxyl groups away from Arg 227, thus creating a rather hydrophobic cavity. The high concentration of positive charges is apparently not well compensated, since it forms only hydrogen bonds with the backbone (carbonyl groups of Ser241). Thus, the mutation of these positions to Met could help to accommodate the hydrophobic environment, as well as to decrease the repulsion of monomers due to uncompensated positive charges.

Mutagenesis and Purification of Mutants

The highest ranking mutant from each of the four sets was selected for further experimental analysis (Table 2). A mutant (C1) combining the mutations of M1 and M3 was also

constructed. All the designed TRAIL mutants were expressed in *E. coli* and purified successfully with a protein yield of ~ 0.7-2 mg/l. Far-UV CD wavelength spectra indicated that all mutants were properly folded with characteristics of a β-sheet containing protein, similar to that of rhTRAIL WT. Gel-filtration and dynamic light scattering measurements showed that all mutant protein solutions contained a single molecule species, consistent with a trimeric oligomerization state. Analytical ultracentrifugation with rhTRAIL WT and M1 corroborated this finding (data not shown).

Table 2 Computational design results

	$\Delta\Delta G_{stability}$ *	$\Delta\Delta G_{binding}$ *‡	**Set**	**Mutations**
M1	-9.7	0.4	Monomer	E194I, I196S
M2	-4.0	7.3	Dimer	D203I, Q205M, Y237F
M3	-7.0	-0.5	Misc.	S225A
M4	-9.1	-1.2	Trimer	R227M
C1	-11.4	-0.9	Combination	M1+M3

* Energy in kcal mol^{-1}, calculated per monomer

‡ $\Delta G_{binding} = \Delta G_{complex} - (\Sigma \Delta G_{chain})$; $\Delta\Delta G_{binding} = \Delta G_{binding}$ mutant $- \Delta G_{binding}$ wild-type

Thermal unfolding

The thermal unfolding of rhTRAIL WT and TRAIL mutants was monitored by CD, measuring changes in molar ellipticity at 222 nm upon heating. Figure 3 shows the heat induced changes of rhTRAIL WT and TRAIL mutants. TRAIL shows an onset of unfolding at approximately 70 °C and has a transition midpoint of 77 °C. The TRAIL mutants show however, onset of unfolding at increased temperatures and higher transition midpoints (Figure 3). For M1 the onset of unfolding was at approximately 76 ° C and the transition midpoint was at 85 °C. M2 showed an onset of unfolding at approximately 74 °C. M3 gave intermediate values between those of rhTRAIL WT and M1, with an onset of unfolding of 73 °C and a transition midpoint of 80 °C. Mutant C1, representing the combined mutations of M1 and M3 showed values comparable to that of M1. The mutant belonging to the trimeric set (M4), however, showed an experimentally determined stability of approximately 3 ºC less than rhTRAIL WT, and was therefore discontinued. The initial increase in molar ellipticity around 76 °C for M2 is due to an overall change of the far UV spectrum, reflecting a loss of structural properties of the starting material (data not shown). Upon cooling all protein solutions were turbid, indicating irreversible aggregation, therefore no thermodynamic parameters could be derived. Far and near UV wavelength CD scans at increasing temperatures confirmed the above findings (data not shown).

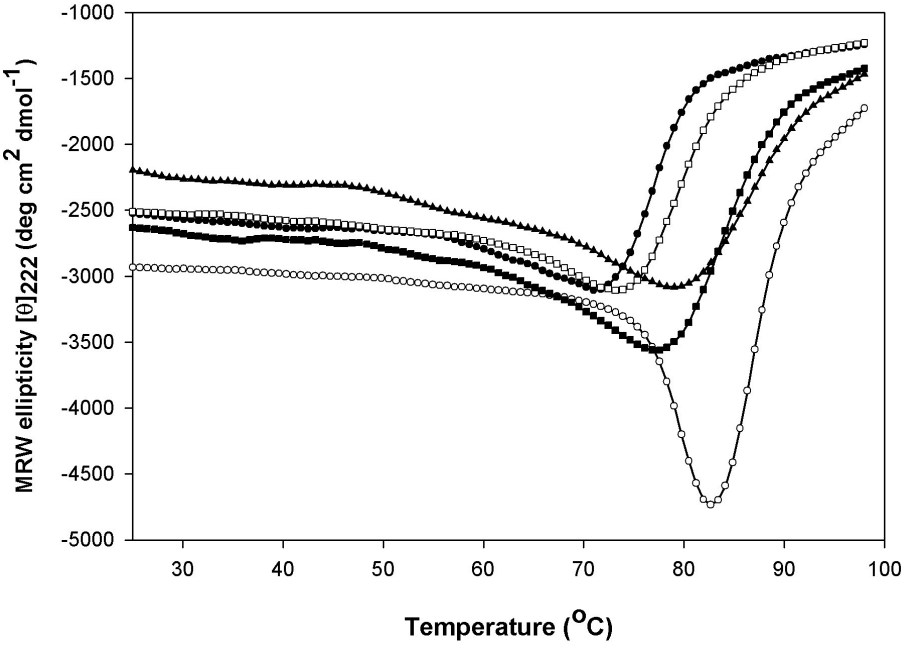

Figure 3. Thermal denaturation profiles of rhTRAIL WT (closed circles), M1 (closed squares), M2 (open squares), M3 (open squares) and C1 (closed triangles).

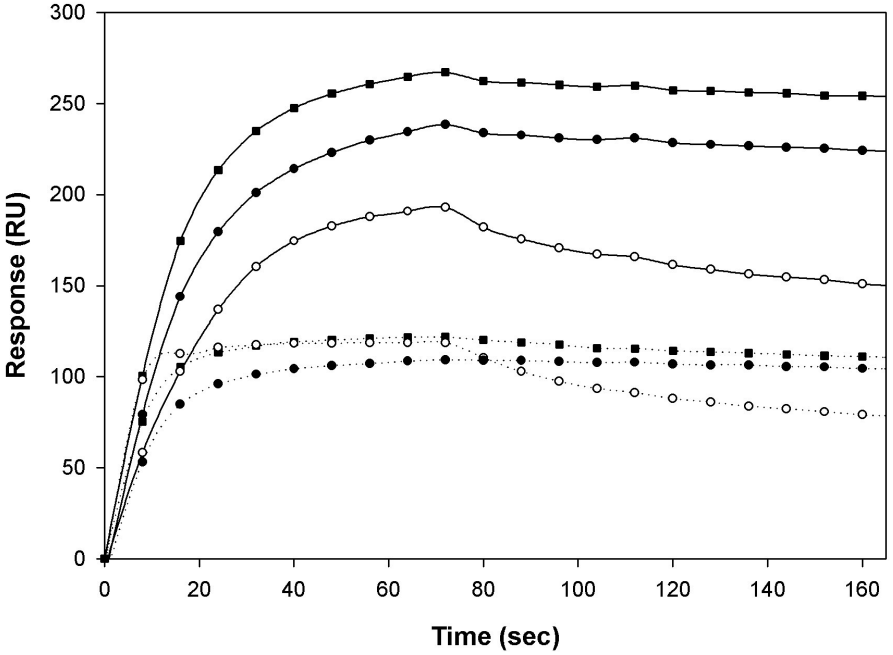

Figure 4. Binding of rhTRAIL WT (closed circles), M1 (closed squares) and M2 (open circles) to DR5 (dotted lines) and DR4 (solid lines) receptors.

***In vitro* bioactivity and binding of designed mutants**

Bioactivity of the TRAIL mutants was assessed *in vitro* using the Colo205 human colon cancer cell line with a MTT based viability assay. A reduction in viability was measured using increasing concentrations of rhTRAIL WT or TRAIL mutants relative to the control. While M1, M3 and C1 showed a bioactivity comparable to that of rhTRAIL WT (ED_{50} ~5 ng/ml), M2 exhibited bioactivity of nearly one order of magnitude lower (ED_{50} ~50 ng/ml). Real-time binding of rhTRAIL WT and TRAIL mutants to the death receptors DR4 and DR5 was assessed using surface plasmon resonance with a Biacore 3000 instrument. Sensorgrams of M1, M3 and C1 were identical to that of rhTRAIL WT. In contrast M2, whilst showing a similar level of binding to both receptors, displayed an increased off-rate when compared to the rhTRAIL WT sensorgram (Figure 4).

Accelerated thermal stability study

In order to test the stability of TRAIL and TRAIL mutants over time, an accelerated thermal stability measurement was performed. The temperature of 73 ^{o} C was chosen to measure effects on stability within a 1 h timeframe. At this temperature rhTRAIL WT starts to unfold, while the mutants are still properly folded (Figure 3). Protein solutions with the same concentration as used in the thermal unfolding measurements were incubated at 73 ^{o}C for 1 h and changes in molar ellipticity at 222 nm were measured (Figure 5). The ellipticity of rhTRAIL WT decreased from the onset, giving a half-life of approximately 13 min. The signal for the M1, M2 and C1 mutants remained essentially constant, indicating an increased thermal stability. M3 showed a half-life of approximately 24 min. These measurements, however, are not indicative of the bioactive trimeric structure of the TRAIL molecule, but of the secondary structure of the monomeric unit. To monitor a concomitant increase in biological activity at elevated temperatures of the mutants with unchanged biological activity (M1, M3 and C1), protein solutions with the same concentrations as used in the thermal unfolding measurements were incubated at 73 ^{o} C and samples were taken at regular intervals for 1 h. Samples were subsequently diluted in tissue culture medium and added to Colo205 cells, resulting in a final concentration of 100 ng/ml. After overnight incubation the viability of the cells was measured using a MTT assay. RhTRAIL WT showed decrease in bioactivity after 20 min of incubation, while M1 and C1 retained full bioactivity after incubation at 73 ^{o} C for 1 h (Figure 6). M3 displayed an intermediate bioactivity between rhTRAIL WT and the other mutants. The increases in thermal stability of the mutants as measured with CD could therefore be correlated with a more stable biologically active trimeric molecule.

Discussion

Others have previously applied computational engineering techniques to improve thermal stability of alpha-helical proteins or monomeric beta-sheet molecules[55,169,170]. However, frequently, monomeric proteins of less than 100 amino acids were used as targets. To our knowledge, this report is the first example of computational redesign of a large trimeric all-β-sheet protein towards a more thermal stable variant. Significantly, it shows that the principles learned from design and engineering of small proteins can also be applied for large multimeric protein complexes.

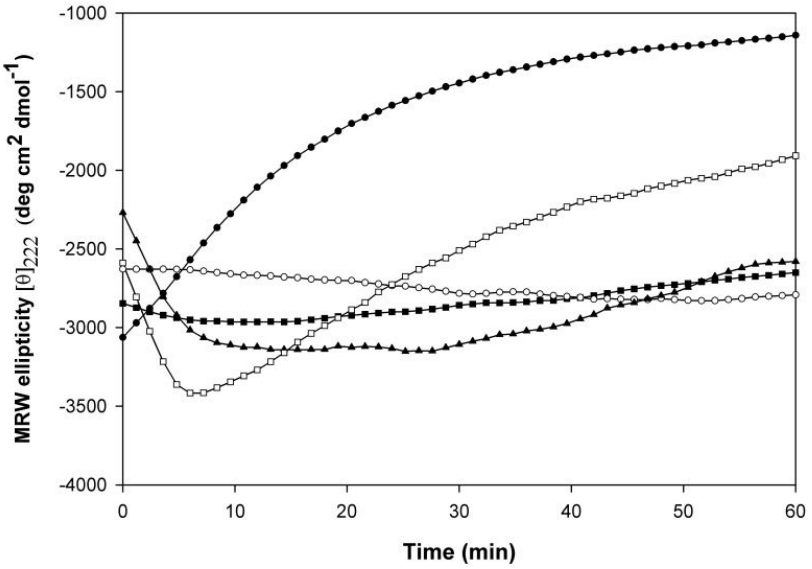

Figure 5. Stability of rhTRAIL WT (closed circles), M1(closed squares), M2 (open circles), M3 (open squares) and C1 (closed triangles) at 73 ºC for 60 min.

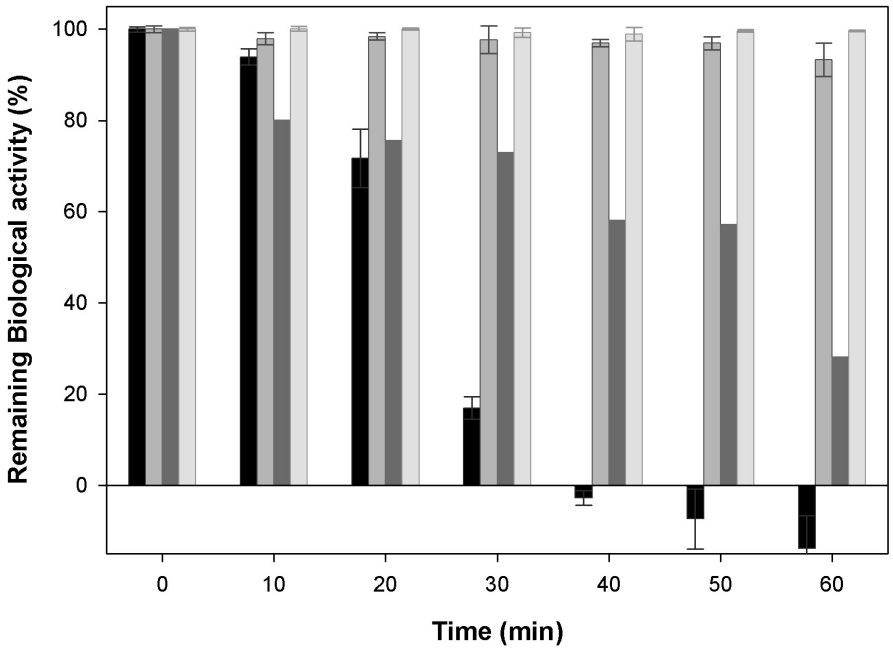

Figure 6. Remaining biological activity of rhTRAIL WT, M1, M3 and C1 (from left to right) upon incubation at 73 º C during 60 min. Biological activities are calculated relative to the value observed at 0 min.

The rhTRAIL WT (114-281) molecule has a relatively high thermal stability if compared to some members of the TNF ligand family. Human tumor necrosis factor alpha (TNF-α), for example, has an apparent T_m of 65 °C as measured with CD[171] and the CD40L receptor binding domain has an apparent T_m of 60 °C as measured with differential scanning calorimetry (DSC)[172]. In parallel investigations, we can show using CD that RANKL however, is more thermal stable than TRAIL, with an apparent T_m of 5 °C higher than rhTRAIL WT, confirming another study[173]. In this study, we investigated the possibility of further increasing the thermal stability of TRAIL, as a model for all-β-sheet proteins, through the use of computational engineering.

We succeeded in extending the thermal stability of the β-sheet protein by more than 5 °C by using a combined approach, employing both TNF ligand family alignment information and an automated computational design algorithm. Due to the non-reversible nature of the unfolding reaction, the apparent T_m is not a perfect indication of an increase in stability. From a functional point of view, therefore, it also makes sense to study the time taken for the protein to denature at high temperature and to relate this to an effect on biological activity. The accelerated thermal stability study showed that the increase in thermal stability of the mutants as measured with CD spectroscopy (Figure 5) can be correlated with the preservation of overall structural characteristics as highlighted by the lasting bioactivity of M1 during the experimental timeframe (Figure 6). When measuring the residual bioactivity of rhTRAIL WT and TRAIL mutants upon incubation at 73 °C for 1 h, it was shown that, while rhTRAIL WT was all but thermally inactivated after ~20 min, the mutants, significantly, had an improved stability with respect to rhTRAIL WT (Figure 5). According to the Arrhenius equation a measured increase in stability for M1 at 73 °C could also correlate with an increase in stability for M1 at more relevant temperatures, such as 37 °C or room temperature, provided that the type of degradation mechanism is the same at both temperatures. Although not tested in this study, it has been shown that in case of certain therapeutically interesting proteins, improvement of thermal stability can also be indicative of an improved *in vivo* half-life[174,175]. This could be of particular interest for the therapeutic use of TRAIL. Preclinical studies showed that rhTRAIL WT was rapidly eliminated from both rodents and non-human primates, with half-lives ranging from 3.6 min (mouse) to 27 min (Chimpanzee)[32].

It is advantageous to use alignment information in order to focus the design on non-conserved residue positions. The reason being that conserved residues are usually retained in a family for a good reason and it is probable that any mutation will decrease protein stability[69,176]. On the other hand, regions with high sequence variability are tolerant to mutation and it can be expected that variants that stabilize the protein can be found in these regions[69]. To accomplish our goal of redesigning a β-sheet protein, TRAIL, and to generate stable variants with the minimum number of mutations, the conserved residues forming the trimeric interface were therefore largely excluded from the prediction/optimization strategy. This resulted in an approach which focused mainly on improvement of the stability of the monomer (intra-chain stabilization; monomeric set) or improving monomer-monomer contacts (inter-chain stabilization; dimeric set). See Table 1.

M1, M2, M3 and C1 showed, in agreement with our predictions, an increase in thermal stability (Table 2; figure 3, 5, 6). Different basic principles were used in the M1, M2 and M3 designs. M1 shows an example of intra-chain stabilization. Stabilization of the flexible CD loop at the surface of each TRAIL monomer results in an increased stability of the entire trimer. This loop is not directly involved in receptor binding and is disordered in un-complexed rhTRAIL WT structures[17,163], but becomes ordered on binding to DR5[12,16,18]. M2, however, illustrates the optimization of the interactions between two adjacent monomers, i.e. inter-chain stabilization. Although we were successful with the above designs, in other cases like the combination mutant, C1 (M1 and M3 combined) or the M4 mutant, we failed in our predictions. There could be several reasons behind it, but it also shows the limitations of design methods. Inherent limitations on force fields, resolution of the structures used as templates and the omission of protein dynamics in the exercise are some of the factors behind protein design failures.

The increase in thermal stability did not affect the biological activity of M1, M3 and C1. M2 was more stable than rhTRAIL WT but the formation of an electrostatic interaction between Gln 205 and Arg 154 of the DR5 receptor was prevented (Figure 6b). This resulted in a subsequent 10-fold decrease in biological activity when compared to rhTRAIL WT, as predicted by FOLD-X ($\Delta\Delta G_{binding}$= 7.3 kcal mol^{-1} $monomer^{-1}$). Our findings confirmed an earlier study showing decreased bioactivity of alanine mutants at these positions[17]. Analysis of binding to the DR4 and DR5 receptors, using surface plasmon resonance, shows an increased off-rate for M2, indicating a lower affinity for both receptors, when compared to rhTRAIL WT and M1 (Figure 2). Since ligand-receptor binding sites are normally "high energy regions", the M2 mutations were expected to stabilize the TRAIL molecule. Thus, this could be regarded as an example of a possible increase in stability which is counterbalanced in evolution by loss of function.

Frequently, other computational redesign studies limited the screening for improvement of thermal stability to the core of the molecule[59,174,175]. Here we show that computational redesign techniques can also involve inter-chain interfaces and surface residues of the molecule, to successfully stabilize the structure.

Performance of PERLA/FOLD-X was successful in the case of the intra-chain (monomer) set, the inter-chain (dimeric) set and the miscellaneous set. The experimental data corresponding to these designs showed all variants within these sets were more stable than rhTRAIL WT. Significantly, we could show that stabilization of the CD loop in a single monomer resulted in stabilization of the entire trimeric molecule (Figure 2a).

Our studies have shown that computer redesign of a more thermal stable multimeric all β-sheet protein is achievable. Computational protein redesign is therefore a valuable addition to other protein engineering methodologies, such as directed evolution or experimental high throughput approaches, as a tool for the improvement of protein properties. Since the computational method used in our study is general applicable, our findings can be further applied to design other TNF ligand family members with improved thermal stability.

Methods

All reagents were of analytical grade unless specified otherwise. Isopropyl-β-D-1-thiogalactoside (IPTG), ampicillin and dithiotreitol (DTT) were from Duchefa. Chromatographic columns and media were from Amersham Biosciences. Restriction enzymes used were purchased from New England Biolabs. All other chemicals were from Sigma.

Computational design of mutants

A detailed description of the protein design algorithm, PERLA, is available elsewhere[159] (http://ProteinDesign.EMBL-Heidelberg.DE) and its use has been previously described[60,85,158,165]. In the case of oligomeric proteins such as TRAIL, protein design requires the following steps: Firstly, residues of a monomer that could establish specific interactions with the contiguous monomer must be identified and selected. Secondly, side chains that contact the residues to be mutated, must be identified to allow side chain movements that are necessary to accommodate the new residues introduced by the algorithm. The algorithm automatically selects these residues based on a geometrical approach that takes Cα-Cα distances and the angle between Cα-Cβ vectors into consideration. Thirdly, the algorithm places the amino acid repertoire at each position selected from a set of naturally occurring amino acids in a multiple sequence alignment of the TNF ligand family, and eliminates those side chain conformations and amino acids that are not compatible with the rest of the structure. Fourthly, all possible pair-wise interactions are explored to eliminate those combinations that are less favorable. Finally, an output of sequences and PDB coordinates corresponding to the best calculated solution (in terms of energy) is produced. The resultant PDB files containing the mutations were energy minimized using GROMOS 43B1 as implemented in Swiss-PdbViewer v3.7b2[177], and evaluated by FOLD-X[160] (http://fold-x.embl-heidelberg.de). The final energies of the models are compared to the reference rhTRAIL WT structure and expressed as ΔΔG (kcal mol^{-1}).

Cloning and PCR

cDNA corresponding to human soluble TRAIL (aa 114-281) was cloned in pET15B (Novagen) using *Nco*I and *BamH*I restriction sites. The N-terminal sequence encoding a His-tag and protease recognition site was therefore removed. Mutants were constructed by PCR using the Quick Change Method (Stratagene) or a modified megaprimer method[178]. The polymerase used was *Pfu* Turbo supplied by Stratagene. Purified mutagenic oligonucleotides were obtained from Invitrogen. Introduction of mutations was confirmed by DNA sequencing.

Expression and purification of rhTRAIL WT and mutants

The rhTRAIL WT and TRAIL mutant constructs were transformed to *Escherichia Coli* BL21 (DE3) (Invitrogen). RhTRAIL WT and M1 were grown at a 5 l batch scale in a 7.5 l fermentor (Applicon) using 4 x LB medium, 1 % (w/v) glucose, 100 µg/ml ampicillin and additional trace elements. The culture was grown to mid-log phase at 37 °C, 30 % oxygen saturation and subsequently induced with 1 mM IPTG. $ZnSO_4$ was added at a concentration of 100 µM to promote trimer formation. Temperature was lowered to 28 °C and the culture

was grown until stationary phase. Other mutants were grown in shake flasks at a 1 l scale at 250 rpm, using a similar protocol. Protein expression was induced when the culture reached OD_{600} 0.5 and induction was continued for 5 h. In this case, the medium used was 2 x LB without additional trace elements.

The isolated pellet was resuspended in 3 volumes extraction buffer (PBS pH 8, 10% (v/v) glycerol, 7 mM β-mercapto-ethanol). Cells were disrupted using sonication and extracts were clarified by centrifugation at 40,000 g. Subsequently, the supernatant was loaded on a nickel-charged IMAC Sepharose fast-flow column and rhTRAIL WT and TRAIL mutants were purified as described by Hymowitz[17] with the following modifications: 10 % (v/v) glycerol and a minimal concentration of 100 mM NaCl were used in all buffers. This prevented aggregation during purification. After the IMAC fractionation step, 20 μM $ZnSO_4$ and 5 mM of DTT (instead of β-mercapto-ethanol) was added in all buffers. Finally, a gelfiltration step, using a Hiload Superdex 75 column, was included. Purified proteins were more than 98 % pure as determined using a colloidal Coomasie brilliant blue stained SDS-PAGE gel. Purified protein solutions were flash frozen in liquid nitrogen and stored at -80 ^{o}C.

CD Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-715 CD spectrophotometer (Jasco Inc.) equipped with a PFD350S Peltier temperature control unit (Jasco Inc.). Rectangular quartz cuvettes with a pathlength of 0.2 cm were used. Protein samples were dialyzed against PBS pH 7.3 and adjusted to a final concentration of 100 μg/ml. Wavelength spectra were recorded between 250-205 nm using a 0.2 nm stepsize and 1 nm band-width at 25 ^{o}C. Temperature scans from 25-98 ^{o}C were performed at 222 nm with a scan rate of 40 ^{o}C/h. Thermal decay measurements were performed at 73 ^{o}C for 1 h at 222 nm.

Bioactivity of TRAIL mutants *in vitro*

Bioactivity of rhTRAIL WT and TRAIL mutants was determined using a viability assay according to the manufacturer's instructions (Celltiter Aqueous One, Promega). Colo205 human colon carcinoma cells (ATCC number CCL-222) were cultured in RPMI 1640 Glutamax containing 10 % heat inactivated fetal calf serum and 100 units/ml Penicillin-Streptomycin. All reagents were supplied by Invitrogen. A concentration series of the rhTRAIL WT or TRAIL mutants was made in cell culture medium. Fifty μl of each dilution was added to a 96-well tissue culture micro plate (Greiner) and 100 μl of cell suspension was added, to a final cell number of $1x10^4$ cells/well. Mixtures were incubated for 16 h at 37 ^{o}C under a humidified atmosphere containing 5 % CO_2. Subsequently, 20 μl of MTS reagent was added. Cell viability was determined after 30 min incubation by measuring the absorption at 490 nm.

Receptor binding

Binding experiments were performed using a surface plasmon resonance-based biosensor Biacore 3000 (Biacore AB, Uppsala, Sweden), at 25 ^{o}C. Recombinant receptors were ordered from R&D systems (R&D systems, Minneapolis, MN, USA). Immobilization of the receptors on the sensor surface of a Biacore CM5 sensor chip was performed following

a standard amine coupling procedure according to the manufacturer's instructions. A reference surface was generated simultaneously under the same conditions but without receptor injection and used as a blank to correct for instrument and buffer artifacts. Purified rhTRAIL WT and TRAIL mutants were injected in two-fold at a concentration of 2 µg/ml and at a flow rate of 20 µl/min. Binding of ligands to the receptors was monitored in real-time. The receptor/sensor surface was regenerated using 3 M sodium acetate pH 5.2 injections.

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